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14. ABSTRACT Our study is testing whether pregnancy affects the growth of breast cancer cells that expressed a mutant form of p53, which we found is responsible for the expression of PAPP-A. One of the hypothesis that was raised is that PAPP-A is a pregnancy-dependent oncogene. This year, we begun the characterization of the PAPP-A transgenic mice. The progress made led to a publication that is pending review. In short, PAPP-A was confirmed to be a pregnancy-dependent oncogene. We also made progress on the role of mutant p53. We found that the growth of genetically identical cells carrying a mutation in p53 is accelerated by the passage through pregnancy. The tumors volumes are bigger in the pregnancy group compared to the virgin group. We also found using microarray analysis that there are genes that are up-regulated specifically in the pregnancy group and not in the virgin group. We have begun the characterization of this pregnancy signature. We also obtained mice carrying a p53 mutant knockin in the p53 knockout mice background. We have established a colony and analyzed the effect of pregnancy on mammary tumor formation. So far we found only one mammary tumor in younger females. We are currently aging these mice. In addition, we have collected primary breast cancer sections and pregnancy history of breast cancer patients prior to their surgery. The analysis of this patient cohort is consistent with the data obtained in the PAPP-A transgenic mice and therefore the data is included in the same publication.				
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INTRODUCTION:

The subject of this research is to understand the link between pregnancy and breast cancer. Pregnancy is associated with an immediate but transient increased risk of breast cancer in all women, with the risk peaking within 5 years after pregnancy. Breast cancer arising shortly after pregnancy are most frequently triple-negative breast cancers, which are characterized by a high rate of p53 mutation and by the activation of IGF signaling. The purpose of this research is to begin to understand the role of pregnancy in breast cancer by focusing on the p53-PAPPA-IGFII axis we have discovered and to test our hypothesis that Pregnancy-associated plasma protein A (PAPPA), a protease required for the activation of IGF signaling, is involved.

The scope of this research is 1) to monitor the activation of IGF signaling and test the efficacy of anti-IGF therapy in our established triple-negative cell line model of pregnancy-dependent breast cancers, 2) to dissect the p53^{MUT}-PAPP-A axis in pregnancy-associated mammary and 3) to determine whether PAPP-A expressing breast cancers are associated with increased serum levels of PAPP-A and IGF-II.

BODY:

Task 1: Establish p53^{WT} and p53^{MUT} xenografts from virgin and parous females.

Research accomplishments:

We have created stable clones expressing either p53^{WT} and p53^{MUT} in MDA-MB 157 breast cancer cells, which are null for p53. We found that pregnancy reduces the growth of tumors expressing p53^{WT} but that pregnancy increases the growth of tumor expressing p53^{MUT} (Fig. 1a). We have created p53^{MUT} xenografts from virgin and parous females. MDA-MB231 cells, which express endogenous p53^{MUT}, were injected in the mammary glands of nude mice females and 2 groups were generated virgin group and the pregnant group, in which a male was added for mating. Pregnancies were recorded. Immediately after pregnancy, the pups were removed to allow involution of the mammary glands. Tumor formation was monitored weekly and tumor volumes determined. For the tumor bank, the tissue was immediately frozen in freezing medium to conserve viability. Another part was fixed in 10% formalin and analyzed for histology and yet another part was preserved for Western or qRT-PCR analyses. For the latter, the mammary gland tissue was snap frozen in liquid nitrogen and pulverized into a powder that can then be used either for protein or RNA extraction. The levels of IGF-II and PAPP-A was determined by qRT-PCR since the current antibodies do not recognize mouse proteins. We found that the volume of tumors derived from mice that have been through pregnancies was significantly higher than in the virgin groups (Fig. 1b).

Task 2: Establish the molecular signature of the p53^{WT} and p53^{MUT} xenografts from virgin and parous females.

Research accomplishments:

We have performed microarray analysis of the tumor derived from virgin or parous females. RNA was extracted and hierarchical clustering was done using DNA-Chip Analyzer software. This analysis was performed using the Genomics Core facility at Mount Sinai. We found that several genes distinguish the two groups (Fig. 1c). Our initial analysis revealed a large number of genes that are significantly different between the two groups. We have begun the validation this signature. Our focus is currently on cytoplasmic p21, collagen, phospho-smad2 cyclin G2, SHARP1 and IGFBP-5 (Figs. 1d, 1e). These genes were selected from the large set of genes based on the fact that another group who has studied the protective effect of pregnancy in wild type p53 cells, found these genes as well. The exception being that in our case the reverse effect in mutant p53 cells was observed on these genes. We take the fact that the positive or negative regulation of this small set of genes correlates with the protective or adverse effect of pregnancy as a strong indication that they are key regulators of the effect of pregnancy. In addition, we found that collagen is also very important. We found increased collagen deposition in the pregnancy group (Fig. 1f). Further, when cells are taken from xenografts and put in vitro on plastic the signature is rapidly lost. However, if the cells are plated on collagen coated plates, a difference in the morphology of the cells was observed (Fig. 1g) and in addition the signature is maintained (Fig. 1h).

Task 3: Test the sensitivity of p53^{WT} and p53^{MUT} xenografts from virgin and parous females to anti-IGF therapy (80 mice).

Research accomplishments:

This task has not been started yet

Task 4: Establish the rate of mammary tumor in p53-mutant knock-in mice (40 mice). In this aim, rate of mammary tumor in the p35MUT knock-in mice will be determined in 4 different groups; 1) virgin, 2) following 1 pregnancy, 3) following 2 pregnancies and 4) following 3 pregnancies. Because p53 KI mice can die of lymphoma, the mammary gland will be extracted and transplant into wild type recipient mice.

Research accomplishments:

The colony has been established. Only one tumor has been found so far but the mice are currently aging.

Task 5: Establish and characterize the effect of PAPP-A on the mammary gland.

Research accomplishments:

We have created MMTV-PAPP-A transgenic mice. Major progress has been made toward this task and led to the submission of a publication (Appendix 1). In brief what we showed is that PAPP-A cleaves its substrate IGFBP-5 during involution and results in a drastic delay of involution (Fig. 2). Further, PAPP-A mice show a drastic increased in collagen deposition during involution. Increased collagen deposition is observed during mammary gland involution in PAPP-A transgenic mice and enhances the proteolytic activity of PAPP-A (Fig. 3). We found that PAPP-A transgenic mice form tumors only after pregnancy and that these tumors are characterized by low IGFBP-5 and expression of tumor associated collagen signature (TACS) (Fig. 4). TACS was discovered by the Keely's laboratory. We have established a collaboration with her group to perform this analysis. TACS are scored as 1, 2 or 3. TACS3, as found in our mice, is associated with poor prognosis in humans. Therefore, this result is highly exciting and suggests that the expression of PAPP-A and the resulting elevated IGF signaling favors the formation of TACS 3.

Another publication is expected from the further characterization of another substrate of PAPP-A, IGFBP-4, during prolactin signaling. This analysis is ongoing.

Task 6: Collect 300 tissue samples and serum from breast cancer patients to determine the levels of PAPP-A in the serum and in the tumor sections.

Research accomplishments:

The collection of human samples has been completed. PAPP-A levels in serum as well as in tumor sections of patients were determined. The serum levels were found to be very low and to generate unreliable results. The immunohistochemistry of PAPP-A was also disappointing as a non-specific cross-reacting protein complicates the interpretation of the results. However, using these samples we were able to determine the level of TACS3 in patients who had children and those who never had children. We found elevated TACS3 in the parous group and this analysis was included in our publication (Fig. 5).

KEY RESEARCH ACCOMPLISHMENTS:

- We have determined that tumors derived genetically identical breast cancer cells grow faster following passage through pregnancy. Therefore, pregnancy stimulates the growth of cancer cells.
- We found that several genes are differentially expressed in virgin and parous-derived mammary tumors.
- We have established a p53 knockin mice colony.
- We have established and begun the characterization of the MMTV-PAPP-A transgenic mice.
- We have written and submitted a first manuscript for publication summarizing the analysis of involution in the PAPP-A transgenic mice.
- We have collect samples from patients and have completed our analysis.

REPORTABLE OUTCOMES:

Manuscripts, abstracts, presentations: Yes. A manuscript has been submitted for publication (Appendix 1). Abstracts and seminars describing our results were presented at Mount Sinai and the University of Wisconsin where Dr. Patricia Keely 's laboratory is located.

Licenses applied for or issued: No

Degrees obtained that are supported by this award: No

Development of cell lines, tissue or serum repositories:

Yes, tissues and blood samples were collected from breast cancer patients.

Informatics such as databases and animal models:

Yes, we have established a novel animal model (MMTV-PAPP-A) and we have generated microarray data on the pregnancy versus virgin tumors.

Funding applied for based on work supported by this award: Yes, an RO1 was written and submitted for funding. Funding is pending decision.

Employment or research opportunities applied for: No

CONCLUSION:

In conclusion, we have obtained data supporting our original hypothesis that pregnancy does accelerate the growth of cells carrying a p53 mutation. We have also obtained data suggesting that PAPP-A is a pregnancy-dependent oncogene.

So what? The knowledge gained from this information is far reaching since it may led to the design a therapy targeted specifically at breast cancers that arise shortly after pregnancy and are directly linked to the remarkable tissue remodeling of the breast during pregnancy.

REREFENCES:

Not applicable

Appendix 1:

Post-partum involution unleashes the oncogenic function of Pappalysin-1 (PAPP-A) in the progression of pregnancy-associated breast cancer.

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Post-partum involution represents a pro-tumorigenic phase of the mammary gland, suggesting a role of involution in pregnancy-associated breast cancers (PABC) ¹. Whether PABC exists as a distinct breast cancer sub-type that is characterized by a unique molecular signature remains controversial. Here, we describe the protease Pappalysin-1 (PAPP-A) as an involution-dependent oncogene. We found that mice expressing PAPP-A in their mammary glands exhibit a marked delay in involution. Mammary ducts of transgenic parous animals are characterized by a major increase in collagen. We consequently demonstrate that the presence of collagen facilitates the proteolysis of IGFBP-5, a key mediator of apoptosis during involution and the only known substrate of PAPP-A during involution. Further, expression of PAPP-A promotes mammary tumors exclusively in parous but not in nulliparous females. These tumors frequently display areas of tumor-associated collagen signature 3 (TACS3), a marker of poor prognosis². Validation of these findings was obtained in premenopausal breast cancer patients, where tumors from parous but not nulliparous patients contain high TACS3 and low IGFBP-5. We propose that the identification of PAPP-A as an involution-dependent oncogene may distinguish PABC from other breast cancer sub-types raising the possibility to develop targeted therapy against PABC.

PABC is currently defined as breast cancer arising within 1 to 2 years after pregnancy. Yet, the increased risk of breast cancer peaks at 6 years following pregnancy³. Therefore, PABC arising beyond the 1 to 2 year window may represent the vast majority of cases, giving PABC a much higher incidence rate. While PABC are frequently of the triple negative (TNBC) sub-type^{3,4}, PABC are more aggressive than TNBC in nulliparous women, suggesting that pregnancy confers unique characteristics to these tumors^{3,4}. The putative oncogenes driving the aggressive behavior of PABC remain uncharacterized. Therefore, their identification would not only distinguish the molecular signature of PABC over TNBC but open the possibility to develop therapy targeted at their unique features.

One major breakthrough regarding PABC is the discovery that involution provides an optimal microenvironment for tumorigenesis¹. This study indicated an increase in collagen deposition during involution that is necessary for creating the pro-tumorigenic milieu¹. However, post-involution mammary glands, also rich in collagen, are known to provide protection against tumor formation⁵. Therefore, other events must take place in actively involuting glands to modulate the effect of collagen.

A key mediator of involution is the insulin-like growth factor binding protein 5 (IGFBP-5)⁶⁻⁹. IGFBP-5 is an inhibitor of IGF signaling and is negatively regulated by the protease Pappalysin-1 (PAPP-A)¹⁰⁻¹³. We hypothesize that degradation of IGFBP-5 would extend involution and that PAPP-A may act as an involution-dependent oncogene. In support of this possibility, PAPP-A has been reported to be overexpressed in 30% of breast cancers¹⁴. However, the oncogenic function of PAPP-A is controversial since the PAPP-A antibody exhibits non-specific cross-reactivity in human breast cancer sections¹⁵. To resolve this controversy, we created transgenic mice expressing human PAPP-A in the mammary glands (MMTV-PAPP-A).

The expression of PAPP-A was confirmed by RT-PCR (Fig. 1a) as well as by immunohistochemistry (Fig. 1b). Consistent with the fact that PAPP-A is a secreted protease, staining of PAPP-A was detected in the lumen of the mammary ducts (Fig. 1b). Since involution is implicated in PABC and IGFBP-5 highly expressed during involution, we focused our analysis on involution. Compared to the non-transgenic females, we found a drastic delay in mammary gland involution (Fig. 1c, d). Further, the degradation of IGFBP-5 during involution was confirmed by immunoblotting (Fig. 1e, f). Interestingly, PAPP-A cleavage of IGFBP-5 was restricted to involution since the level of IGFBP-5 in the mammary gland of virgin PAPP-A transgenic mice was similar to that of non-transgenic mice (Fig. 1g, h). This result suggests that involution provides the key elements that allow cleavage of IGFBP-5 by PAPP-A.

One likely event to potentiate IGFBP-5 proteolysis is the deposition of collagen during involution since IGFBP-5 binds to collagen⁶ and IGF signaling increases expression of collagen¹⁶. To test this possibility, involuting mammary glands from non-transgenic and PAPP-A transgenic females were stained with Masson's trichrome to detect collagen. This analysis revealed a substantial increase in the level of collagen staining in the PAPP-A transgenic mice (Fig. 2a). The increase in collagen was confirmed using second harmonic generation (SHG) imaging (Fig. 2b)^{2,17}. This analysis showed a significant increase in the percentage of positive areas in mammary glands of the PAPP-A transgenic females during involution (Fig. 2c) as well as highly significant increase in the deposition of collagen surrounding the ducts (Fig. 2d).

To investigate directly the effect of collagen on the cleavage of IGFBP-5, we took advantage of the observation that recombinant IGFBP-5 (rIGFBP-5) is found in the pellet (P) fraction after low speed centrifugation (Fig. 2e). However, addition of detergent (NP-40) to the reaction allowed detection of rIGFBP-5 in the supernatant (S) suggesting that rIGFBP-5 is prone to aggregation (Fig. 2e). We found that addition of collagen alone without detergent also led to the detection of rIGFBP-5 in the supernatant (Fig. 2e). This result indicates that collagen may affect the folding

of IGFBP-5 and increase its solubility. We then tested the effect of collagen on the ability of PAPP-A to cleave IGFBP-5. For this assay, media from MCF-7 cells or MCF-7 cells stably expressing PAPP-A were used. We found that rIGFBP-5 is resistant to cleavage by PAPP-A in the absence of collagen (Fig. 2f). Upon addition of collagen however, PAPP-A degraded rIGFBP5 in both fractions (Fig. 2f). To further test this result, media from control cells (MCF7), cells expressing active PAPP-A (wt) and cells expressing proteolytically inactive PAPP-A (mut) were tested on unfractionated rIGFBP-5 without (Fig. 2g, h,) and with (Fig. 2i, j) collagen, as well as on endogenous IGFBP-5 (Fig. 2k, l, m, n). In both cases, we found that addition of collagen increased the degradation of IGFBP-5. Collectively, these results suggest that increased collagen deposition is the necessary element that facilitates cleavage of IGFBP-5 by PAPP-A specifically during involution.

This observation raises the possibility that PAPP-A acts as an involution-dependent oncogene. We therefore examined the frequency of mammary tumors in age-matched virgin versus parous transgenic females. Mammary hyper-proliferative lesions were detected exclusively in the parous groups, while virgin female glands contained no detectable lesions (Fig. 3a, b). Palpable mammary tumors were observed after a latency of 14 months (Fig. 3c, d) and were confirmed to have low expression of IGFBP-5 (Fig. 3d). While the MMTV promoter is inducible by hormones¹⁸, the MMTV promoter used to establish our PAPP-A transgenic mice is a truncated version where the hormonal sensitive region is deleted¹⁸. In agreement with hormonal insensitivity in our model, neither insertion of an estrogen pellet nor involution increased PAPP-A levels (Fig3. f). These results therefore rule out an indirect effect of transgene expression levels.

Having established that PAPP-A transgenic females show an increased deposition of collagen during involution, we next aimed at analyzing tumor-associated collagen signatures (TACS)¹⁹. Importantly, TACS do not only measure the density of collagen but also the orientation of

collagen fibers relative to the tumor border. TACS are classified as TACS1, 2 or 3, where radially oriented collagen fibers characterize the TACS3 signature and is strongly associated with more aggressive breast cancers². We found TACS3 regions in 50% of the cell-matrix border of the PAPP-A mammary tumors (Fig. 3 g, h). We also determined the frequency of TACS3 in two unrelated MMTV models of breast cancers, the MMTV-HER2 and MMTV-WNT. We found that TACS3 was highly correlated with PAPP-A tumors (Fig. 3 g, h). This finding indicates that the increased deposition of collagen induced by PAPP-A favors the formation of TACS3.

Collectively, our data show that PAPP-A is an involution-dependent oncogene that promotes the formation of mammary tumors characterized by both a TACS3 signature as well as low IGFBP-5 levels. Since immunohistochemistry of PAPP-A cannot be used on human breast cancer due to cross-reactivity¹⁵, to validate this observation in humans, we used TACS3 and IGFBP-5 as markers of PAPP-A overexpression. Specimens were collected from newly diagnosed breast cancer patients in pre-menopausal women. In addition to tissue sections, history of pregnancy was obtained. Two groups of age-matched patients were established, those that were never pregnant and those who had at least one child. TACS3 was determined by SHG microscopy in both groups. We found a significantly higher rate of TACS3 in the parous group relative to the nulliparous group (Fig. 4 a, b, c). Further, we also found that expression of IGFBP-5 was lower in the parous group compared to the nulliparous group (Fig. 4d).

A summary of the mechanism of how PAPP-A acts as a pregnancy-dependent oncogene and may contribute to PABC is shown in figure 4e. While PAPP-A was reported to be overexpressed in 30% of breast cancers¹⁴, the validity of these results were put into question¹⁵. Our results demonstrate that PAPP-A is indeed an oncogene. Further, its oncogenic function is unleashed specifically during involution. Importantly, overexpression of PAPP-A delays involution and therefore amplifies the pro-tumorigenic niche that this developmental phase of the breast

represents. Adding to these effects is our finding that PAPP-A not only promotes further deposition of collagen but favors the reorientation of collagen fibers into the pro-invasive TACS3 signature. How PAPP-A favors the formation of TACS3 is currently unclear. One possibility is that since IGFBP-5 binds to collagen, the proteolysis of IGFBP-5 itself may alter the conformation of collagen. Alternatively, since collagen interacts with several proteoglycans and that PAPP-A contains a proteoglycan binding site, it is tempting to speculate that proteolysis of proteoglycans may also contribute to the realignment of collagen fibers. Nevertheless, the validation of high TACS3 and low IGFBP-5 in parous breast cancer patients suggests that distinguishing markers of PABC can be identified. This possibility remains to be addressed in a larger patient population for which clinical follow-up is available.

Collectively, data presented here identifies PAPP-A as the first involution-dependent oncogene to promote the formation of PABC and therefore help define PABC as a distinct sub-type. Lastly, since several drugs targeting the IGF pathways are tested in the clinic ²⁰, our findings open the possibility to develop targeted therapy for PABC.

Legend to figures:

Figure 1 Expression of PAPP-A in the mammary gland of transgenic mice leads to a delay in mammary gland involution. **(a)** Relative PAPP-A mRNA expression in the mammary gland of 2 6-week-old non-transgenic (NT) mice and 3 mice from 3 different founders of PAPP-A transgenic (PAPP-A) lines. **(b)** Histological sections of mammary glands from 6-week-old female mice, left: H&E and right: PAPP-A immunohistochemistry, scale bar: 50um. **(c)** Whole mount analysis of involuting mammary glands at day 1, 3, 6, 12 of involution in NT and PAPP-A females, scale bar: 1mm. and, **(d)** H&E sections of involuting mammary glands at day 1, 3, 6, 12 of involution in NT and PAPP-A females, scale bar: 100um. **(e)** Immunoblot of IGFBP-5 in mammary glands during involution time course in NT and PAPP-A transgenic mice. **(f)** Quantification of IGFBP-5 from immunoblot shown in (e). **(g)** Immunoblot and quantification of IGFBP- 5 in mammary glands of 6-week-old virgin NT versus PAPP-A transgenic females. **(h)** IGFBP-5 immunohistochemistry in mammary glands from 6-week-old virgin NT versus PAPP-A transgenic mice, scale bar: 50um.

Figure 2 Increased collagen deposition is observed during mammary gland involution in PAPP-A transgenic mice and enhances the proteolytic activity of PAPP-A. **(a)** Masson's trichrome stain (MTS) of involuting mammary glands of NT and PAPP-A transgenic mice. Blue: collagen. Scale bar: 100um. **(b)** Second harmonic generation (SHG) imaging of collagen on histological sections shown in (a). Scale bar: 100um. **(c)** Quantification of collagen by MTS from (a). **(d)** Graph of collagen intensity versus distance from NT and PAPP-A involuted mammary ducts as imaged by SHG in (b). **(e)** 50ng Recombinant IGFBP-5 (rIGFBP-5) fractionated into supernatant (S) and

pellet (P) in the presence of media, collagen (+Col-1), collagen neutralizing solution (+ N.S.), or detergent (+NP40). (f) Protein levels (in S versus P fractions) of 50ng rIGFBP-5 incubated for 3 hours in control media from MCF7 parental cells or PAPP-A media taken from stable MCF7^{PAPP-A} clones, in the absence or presence of collagen. (g) Immunoblot and (h) quantification of 50ng rIGFBP-5 incubated for 3 hours in culture media from MCF7 parental cells, MCF7 clones stably expressing wild-type PAPP-A (MCF7^{wtPAPP-A}) or proteolytically inactive PAPP-A (MCF7^{mutPAPP-A}) in the absence of collagen. (i) Immunoblot and (j) quantification in a corresponding assay as in (h) in the presence of collagen. (k) Immunoblot and (l) quantification of endogenous IGFBP-5 levels of parental MCF7, MCF7^{wtPAPP-A} stable clones or MCF7^{mutPAPP-A} stable clones, in the absence of collagen. (m) Immunoblot and (n) quantification in a corresponding assay as in (l) in the presence of collagen.

Figure 3 Pregnancy-associated PAPP-A mammary tumors are characterized by low IGFBP-5 levels and a TACS3 signature. (a) Whole mount sections of PAPP-A mammary glands after 1 or 3 pregnancies and PAPP-A age-matched virgin. White arrows indicate lesions. Scale bar: 1mm. (b) Frequency of mammary lesions in NT virgin/pregnancy and PAPP-A virgin/pregnancy glands, n = 4 in each group. (c) Image of a late-stage post-partum PAPP-A mammary tumor before excision. (d) Histological sections of tumor from (c), left: H&E, center: IGFBP-5 immunohistochemistry, right: IGFBP-5 IHC of NT involuted gland. Scale bar: 100um. (e) Relative PAPP-A mRNA in mammary glands of NT virgin, PAPP-A virgin, and a PAPP-A virgin 1 week after injection of estrogen pellet. (f) Relative PAPP-A mRNA in mammary glands of virgin, or involuting glands at day 1 or day 12 in NT versus PAPP-A transgenic females. (g) Imaging of collagen by SHG of virgin PAPP-A ducts or PAPP-A mammary tumor, and mammary tumors

from MMTV-HER2 or –WNT transgenic models. Scale bar: 50um. (h) TACS3 analysis for collagen alignment of SHG images from (g).

Figure 4 A strong TACS3 signature and decreased IGFBP-5 levels characterize postpartum breast cancers. (a) SHG image of collagen of human breast cancers from parous and nulliparous patients. The dotted line indicates the tumor-stroma border. Scale bar: 50um. (b) Distribution of collagen curvelet angles, as calculated by CurveAlign software, of breast cancer sections from nulliparous (n=9) and parous (n=14) patients. (c) TACS3 signature score of breast cancer from nulliparous (n = 9) and parous (n = 14) patients, $p < 0.0001$. (d) Representative scoring of immunohistochemistry of IGFBP-5 on invasive breast cancer sections, with score 0 as negative staining, score 1 as weak staining, and score 2 as strong staining. Top scale bar: 100um. Magnified image scale bar: 20um. Table displays distribution of score in nulliparous (n = 10) and parous (n = 12) sections. (e) Schematic diagram of a mechanism by which upregulated PAPP-A cleaves IGFBP-5 (left) and increases binding of IGF to IGF receptors, resulting in increased IGF signaling. In addition to maintaining proliferation, elevated IGF signaling promotes the synthesis of collagen (right), which in turn facilitates IGFBP-5 proteolysis by PAPP-A, establishing a positive feedback loop. Since increased deposition of collagen in breast cancers from parous women is characterized by a TACS3 signature, which stimulates invasion, we hypothesize that low IGFBP5 and strong TACS3 contribute to the more aggressive nature of PABC.

Acknowledgments: We thank Jaisa Perez for collecting patient samples and the staff of the Dubin Breast Center. We also thank Corinne Vokoun, Jeremy Bredfeldt, Yuming Liu and Matthew Conklin for advice and input on SHG imaging and analysis. This work is supported by a Breast Cancer Program, Idea Award W81XWH-12-1-0060 from the Department of Defense to D.G.

ONLINE METHODS

Animal procedures. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Mice overexpressing *PAPP-A* in the mammary gland (MMTV-PAPP-A) in a FVB/n background were generated as follows: The PAPP-A wild-type cDNA was cloned into an expression plasmid containing the mouse mammary tumor virus (MMTV) long terminal repeat plus simian virus 40 intron and polyadenylation signals. A linearized MMTV-PAPP-A construct was microinjected into FVB/n mouse oocytes at the Mount Sinai Mouse Genetics Shared Research Facility and both female and male founders were identified by genotyping of tail genomic DNA. Animals were sacrificed by CO₂ followed by cervical dislocation, and tissues for biochemical analyses were frozen at -80 degrees (for tissue analysis protocols, see 'Whole-mount analysis' and 'Histology and immunohistochemistry'). For involution time-course experiments, pups were separated from mothers two days after birth to allow for lactation (day 0) and females were sacrificed at day 1, 3, 6 or 12 as described above. For estrogen treatment, a 0.72mg/ml 17 beta-estradiol pellet (Innovative Research of America, Cat#SE-121) was inserted in the cervical region of an adult virgin MMTV-PAPP-A female. The animal was maintained for 1 week before harvesting mammary glands.

RNA Extraction and Real Time RT-PCR. Total RNA was extracted from cell lines and mammary tissues using RNeasy Plus Mini Kit (cat. No. 74134). 100ng of each sample was used in real-time RT-PCR reaction using Takara One-Step Primescript RT-PCR Kit (Cat# RR064A) following the manufacturer's protocols. Human PAPP-A primers (forward: 5'-

GTCAATGTTTCCTTCCAGTGC-3' and reverse: 5'-CTTGTGCTTATTCTCTCGGGC-3') were used in the study along with the beta-actin primers as loading control.

Whole-mount analysis. Mouse mammary glands were dissected and spread onto glass slides and fixed in Carnoy's fixative (6 parts absolute ethanol, 3 parts chloroform, 1 part glacial acetic acid) overnight. Mammary glands were placed in 70% ethanol for 15 minutes, with gradual exchange in MilliQ H₂O before incubation in carmine aluminum stain overnight. Slides were stored in 70% ethanol and imaged at 1x magnification under a light microscope.

Histology and immunohistochemistry. Histological slides were prepared as 4 micron formalin-fixed sections embedded in paraffin and stained with H&E by the Oncological Sciences department histology core facility at Mount Sinai School of Medicine. Masson's trichrome staining (DAKO, Cat#AR173) of sections was performed following manufacturer's instructions. Images of stained sections were collected in triplicates of representative areas at 20x magnification. The blue stain was manually selected for and converted to black with Adobe Photoshop CS5's (Version 12.0 x64) magic wand tool. The images were opened in ImageJ (Version 1.46r) and the black selections were quantified as % area positive for collagen. For immunostaining of paraffin-embedded sections, samples were dewaxed and rehydrated in water, and antigen retrieval was performed in citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes. All buffer washes between antibody incubations were with 50 mM Tris-HCl, pH 7.6. Primary antibodies: PAPP-A rabbit polyclonal antibody (DakoCytomation, Cat#A0230, 1:250) and IGFBP-5 rabbit polyclonal antibody (Santa Cruz, Cat#sc-13093, 1:50). Primary antibody incubation was performed for 1 hour, RT and detected with the LSAB+ kit peroxidase (DAKO, Cat#K0690) with biotinylated anti-rabbit IgG for

1 hour and streptavidin peroxidase for 10 minutes. Slides were developed with AEC (3-amino-9-ethyl-carbazole) chromogen (DAKO, Cat#K0690) for 7 - 10 minutes. Sections were then counterstained in haematoxylin and mounted with Permount (Fischer Scientific, Cat#SP15-100).

Immunoblot. Mammary glands were homogenized in NP-40 lysis buffer (50mM Tris, 250mM NaCl, 5mM EDTA, 0.5% NP-40, 50mM NaF, 1mM DTT) and 30µg total protein in Laemmli buffer per sample was loaded on SDS-glycine polyacrylamide gels ran at 80V for 30 minutes, 200V for 45 minutes. Proteins were transferred to nitrocellulose membranes for 1 hour at 80V. Membranes were blocked in 4% milk in TBS-T and incubated on a rotator overnight at 4°C in the following primary antibodies: IGFBP-5 (Upstate, Cat#06-110, 1:1,000), Actin (Chemicon Int'l, Cat#1378996, 1: 20,000) and GAPDH (Calbiochem, Cat#CB1001, 1: 20,000). After three washes in TBS-T, membranes were incubated in secondary rabbit or mouse antibodies coupled to peroxidase (Jackson ImmunoResearch, Cat#115-035-146/111-035-146, 1:5,000) in 2% milk/TBS-T for 1 hour, RT. Signal detection was performed with ECL (GE healthcare, Cat#RPN2106) following manufacturer's protocols.

Second-harmonic generation imaging of mouse samples and analyses. All mouse mammary gland SHG images were captured on an Olympus FV1000MPE Fluoview multi-photon microscope (Tokyo, Japan) at the Mount Sinai School of Medicine microscopy core facility. Specs for imaging include a Coherent Chameleon Vision II Ti:S laser (Santa Clara, CA) with a 680 – 1080 nm tuning range, dispersion compensation, and 140 fs pulse width. An Olympus XLPlanN 25X/1.05 numerical aperture water immersion lens was used for image acquisition. The excitation wavelength was set at 900 nm, and signal collection was performed by backwards imaging with a 420 – 460nm bandpass filter, a 485 dichroic mirror (GR/XR filter

cube, Olympus), and an external detector. Images were acquired at a 1024 x 1024 pixel resolution while keeping the power constant throughout. Collagen intensity versus distance analyses were performed in six replicate ducts per mouse in three mice per group (NT versus PAPP-A) following a protocol described previously¹. Involution duct SHG images were opened in ImageJ (Version 1.46r), and a rectangular selection of 50 x 100 pixels was made bordering the edge of ducts in four directions. The four regions of interest (ROI) were analyzed using the profile plot function, and intensity of the SHG signal over a 40µm distance for the four ROIs was averaged as the signal for a single duct. For tumor-associated collagen signature-3 (TACS3) analyses of SHG images in mice, a 3 x 3 stitched image (3070 x 3070 pixels) was captured in triplicates for two representative slides per group (MMTV-PAPP-A, MMTV-HER2, MMTV-WNT). Each image was divided in 16 sub-regions (767.5 x 767.5 pixel ROIs) and only those containing a tumor border were manually scored for presence of collagen perpendicularly aligned against a tumor border. The results were subsequently displayed in Excel (Microsoft Version 14.1.0) as % TACS3 positive.

Establishment of MCF7-PAPP-A stable clones. MCF7 breast cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. MCF-7^{PAPP-Awt} and MCF-7^{PAPP-Amut} stable clones were generated by transfection of a PAPP-A wild-type (pcDNA3.1-PAPP-A) or mutant (pcDNA3.1-PAPP-A^{E483Q}) construct using Mirus (TransIT-LT-1, Mirus) following manufacturer's instructions. G418-selected stable clones were confirmed for PAPP-A expression by qRT-PCR (see below) and Quantikine PAPP-A ELISA (R & D systems, Cat#DPPA00).

IGFBP-5 solubility and PAPP-A cleavage assays. Collagen gels were formed in Eppendorf tubes at 37°C for 30 minutes by mixing 1:1 rat tail collagen I protein (Gibco, Cat#A1048301) with neutralizing solution (100mM Hepes, PH7.3 in 2x PBS). 50ng of recombinant human IGFBP-5 (r-IGFBP-5) (Abcam, Cat#49835) were co-incubated with 15µl collagen gel at room temperature (RT) for 3 hours in 15µl serum-free DMEM, neutralizing solution, or 0.01% NP40 detergent. Samples were fractionated into supernatant (S) and pellet (P) by centrifugation at 14,000rpm for 12 minutes, RT. Immunoblot samples were prepared with addition of 1x Laemmli sample buffer to 40µl final volume and boiling for 5 minutes. Cell-free cleavage assays were performed using PAPP-A protein secreted in 24-hour-serum-free culture media from MCF-7^{PAPP-Awt} stable clones grown to confluency (in complete media) on 10cm plates. Culture media from parental MCF-7 was used as a negative control for PAPP-A protein in cell-media. 50ng of r-IGFBP5 were co-incubated with 15µl collagen gel at 37°C for 3 hours in either 15µl MCF7 parental media or MCF-7^{PAPP-Awt} media. The assay was inactivated by addition of 1x Laemmli sample buffer to 40µl final volume and boiling for 5 minutes. *In vitro* cleavage of endogenous IGFBP-5 from cell lines were performed by growing MCF-7 parental, MCF-7^{PAPP-Awt} or MCF-7^{PAPP-Amut} on plastic or collagen gels for 3 days. Cells were harvested in 2x Lysis/RIPA buffer (50mM HEPES, PH7.4, 150mM NaCl, 2mM EDTA, 2mM NaF, 2% NP-40, Protease inhibitor cocktail) and prepared for immunoblot in 1x Laemmli sample buffer to 40µl final volume and boiling for 5 minutes.

Collection of patient samples. Collection of pregnancy history and tissue sections from newly diagnosed breast cancer patients was performed according to our IRB approved protocol. Histological sections from patients were obtained from the Department of Pathology at Mount Sinai. In our analysis, patients diagnosed with breast cancer before or at the age of 51 were classified as pre-menopausal.

Scoring of human breast cancer specimen. Multiphoton microscopy for patient specimens was performed at the University of Wisconsin Laboratory for Optical and Computational Instrumentation². Imaging was done on a Prairie Technologies Ultima IV multiphoton microscope at a excitation wavelength of 890 with a Spectra Physics Insight laser. All images were collected with 20X 1.0 NA Objective (Olympus) and using a 445 bandpass filter to discriminate for SHG. SHG images at a resolution of 1024 x 1024 pixels were taken in triplicates, and two tumor border ROIs per image were analyzed using the CurveAlign (Version 2.2 R2012a) software, covering 700 – 1000 collagen fibers (curvelets) analyzed over 6 ROIs per sample. Curvelets with an angle against the border below 40° were assigned a score of 1, between 40 and 60° a score of 2, and above 60° a score of 3. The fraction of curvelets belonging to each of these three angle ranges was multiplied by the respective scores and added to obtain the final TACS3 score per patient. n = 9 for nulliparous, n = 14 for parous. For IGFBP-5 immunohistochemistry, a score of 0 was assigned to negative staining in invasive regions, score 1 to regions with weak staining, and score 2 to regions with strong staining. A section was considered positive for IGFBP-5 if more than 20% of cells displayed a score 2 stain and was highlighted in red in the table in 4d. n = 10 for nulliparous, n = 12 for parous.

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Figure 1:

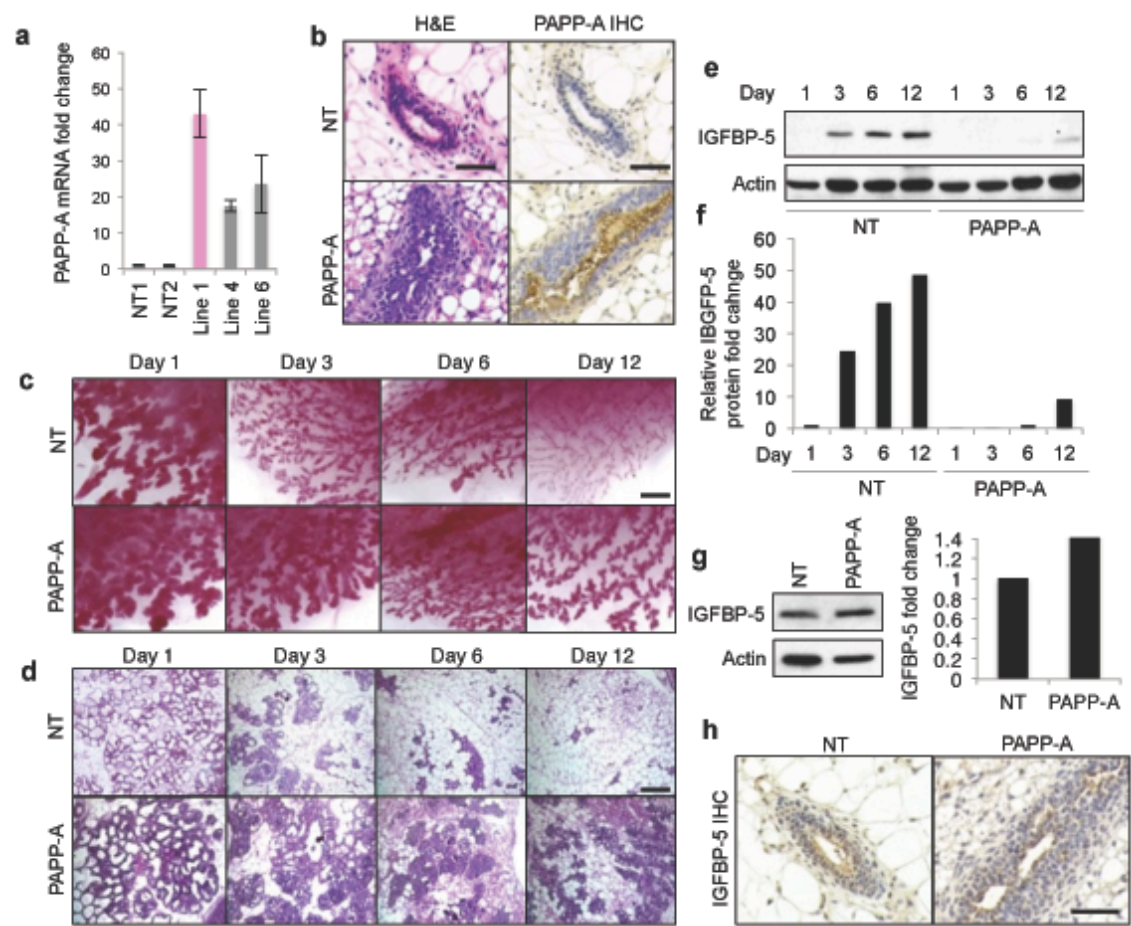


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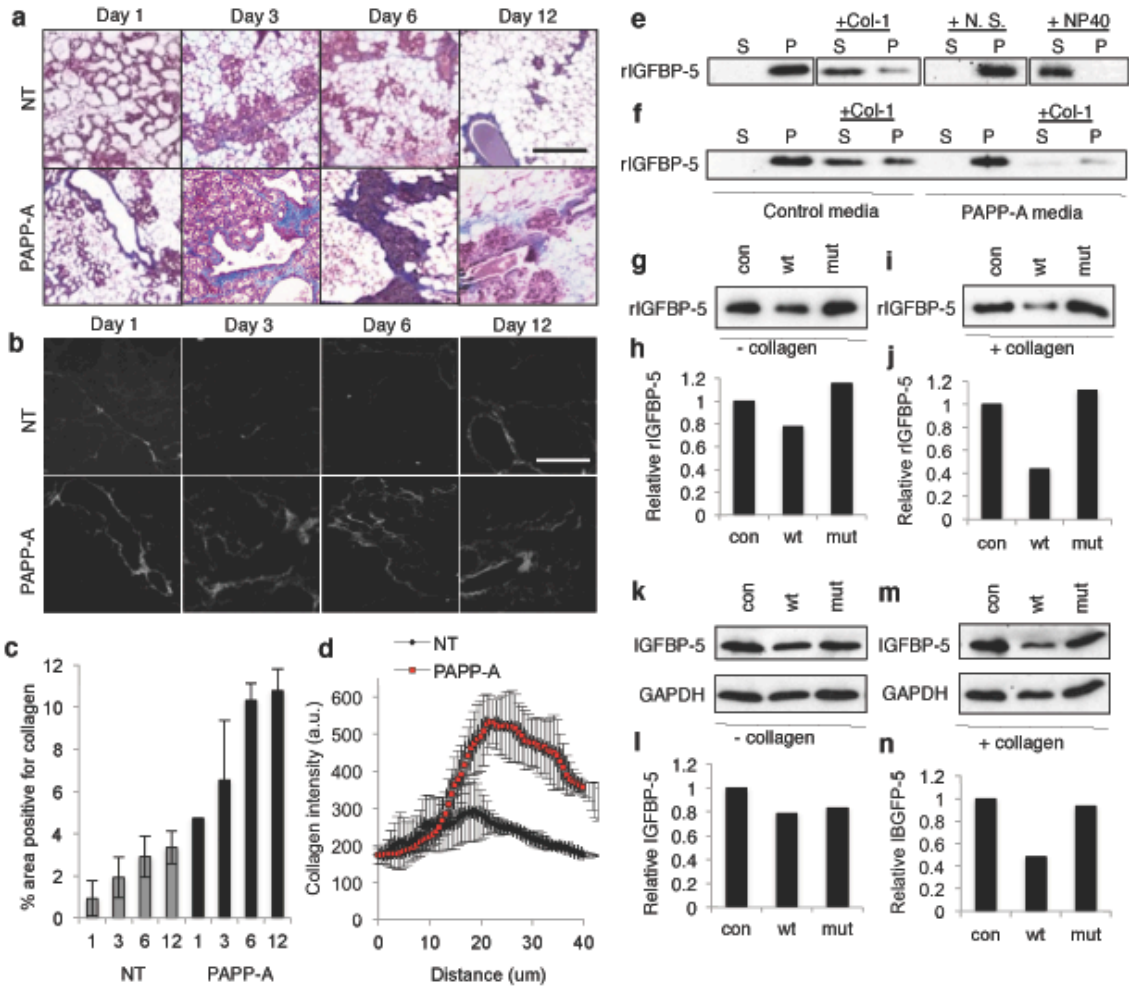


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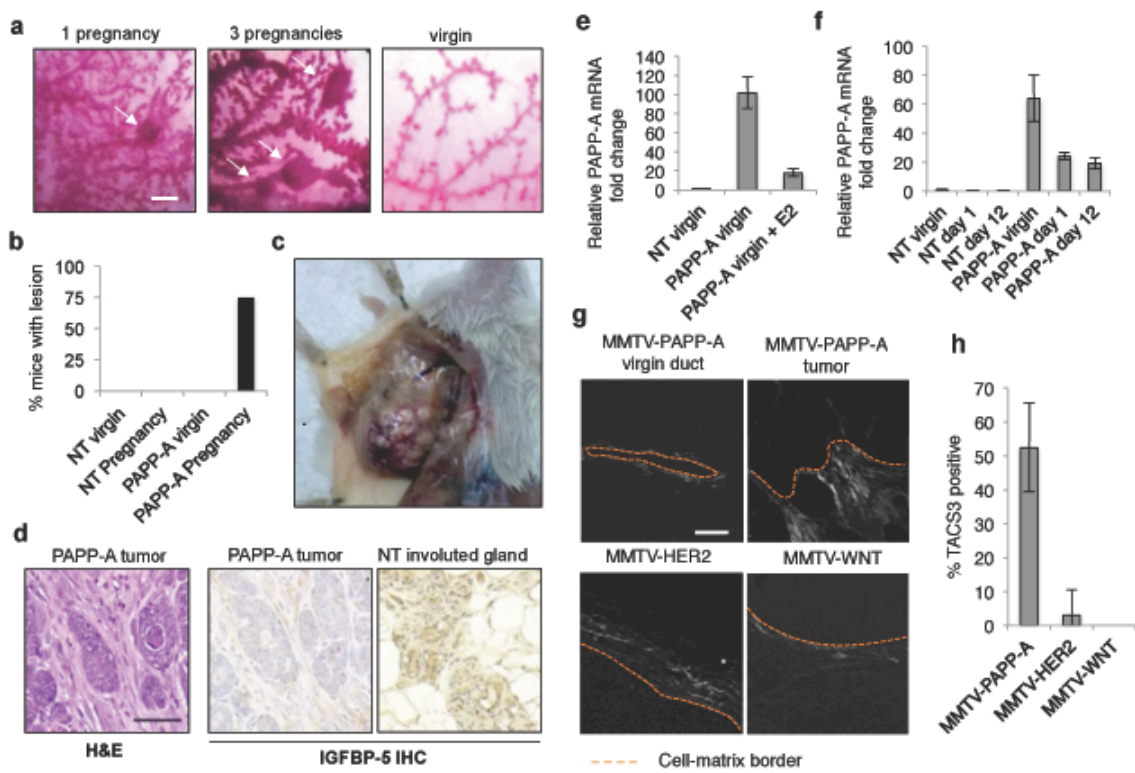
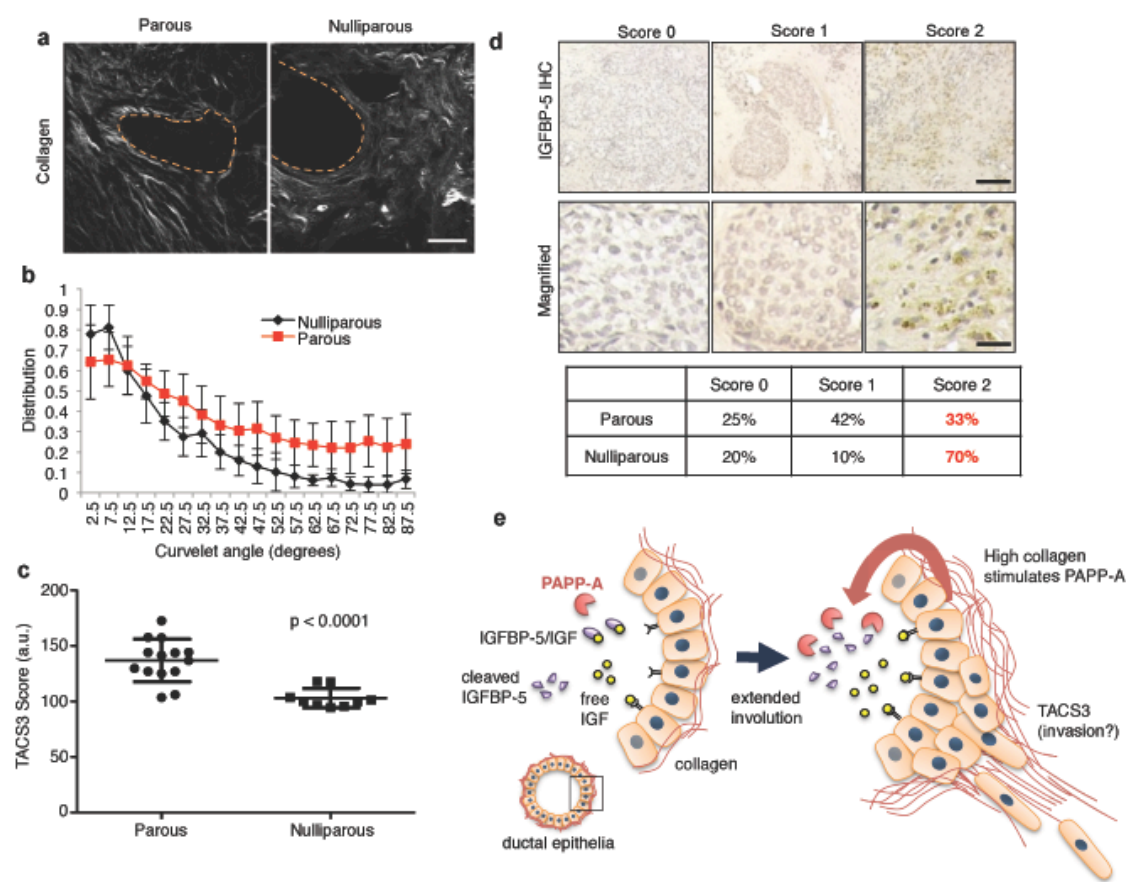


Figure 4:



Appendix 2: Supporting data

Figure 1 Pregnancy-driven MDA-MB-231 xenografts show a unique signature associated with their accelerated growth. A) MDA-MB-157 xenografts wild-type or mutant (157 or 248) for p53 measured for tumor size in virgin vs. pregnancy. B) MDA-MB-231 xenografts growth rate in virgin versus pregnancy. C) Microarray of gene sets upregulated and downregulated in virgin versus pregnancy MDA-MB-231 xenografts; D) MDA-MB-231 xenograft were used for western analysis of the indicated protein. E) MDA-MB-231 xenograft mRNA of Cyclin G1 and SHARP1. F) MDA-MB-231 xenograft collagen staining. G) collagen *ex vivo* culture of MDA-MB-231 xenografts at 5 days. H) Western of indicated proteins from virgin-derived or pregnancy-derived cells grown on collagen *ex vivo* for 5 days. I) p21 nuclear vs. cytoplasmic protein levels in MDA-MB-231 xenografts. V indicates virgin, P indicates parous.

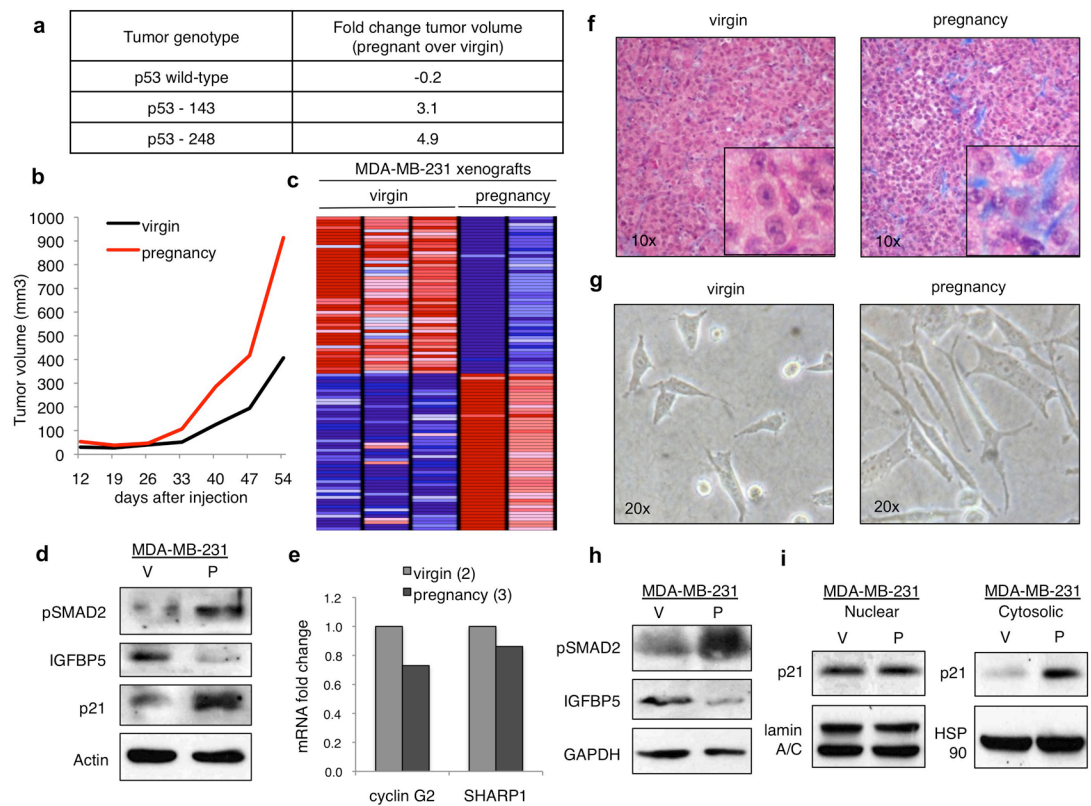


Figure 2 Expression of PAPP-A in the mammary gland of transgenic mice leads to a delay in mammary gland involution. **(a)** Relative PAPP-A mRNA expression in the mammary gland of 2 6-week-old non-transgenic (NT) mice and 3 mice from 3 different founders of PAPP-A transgenic (PAPP-A) lines. **(b)** Histological sections of mammary glands from 6-week-old female mice, left: H&E and right: PAPP-A immunohistochemistry, scale bar: 50um. **(c)** Whole mount analysis of involuting mammary glands at day 1, 3, 6, 12 of involution in NT and PAPP-A females, scale bar: 1mm. and, **(d)** H&E sections of involuting mammary glands at day 1, 3, 6, 12 of involution in NT and PAPP-A females, scale bar: 100um. **(e)** Immunoblot of IGFBP-5 in mammary glands during involution time course in NT and PAPP-A transgenic mice. **(f)** Quantification of IGFBP-5 from immunoblot shown in (e). **(g)** Immunoblot and quantification of IGFBP-5 in mammary glands of 6-week-old virgin NT versus PAPP-A transgenic females. **(h)** IGFBP-5 immunohistochemistry in mammary glands from 6-week-old virgin NT versus PAPP-A transgenic mice, scale bar: 50um.

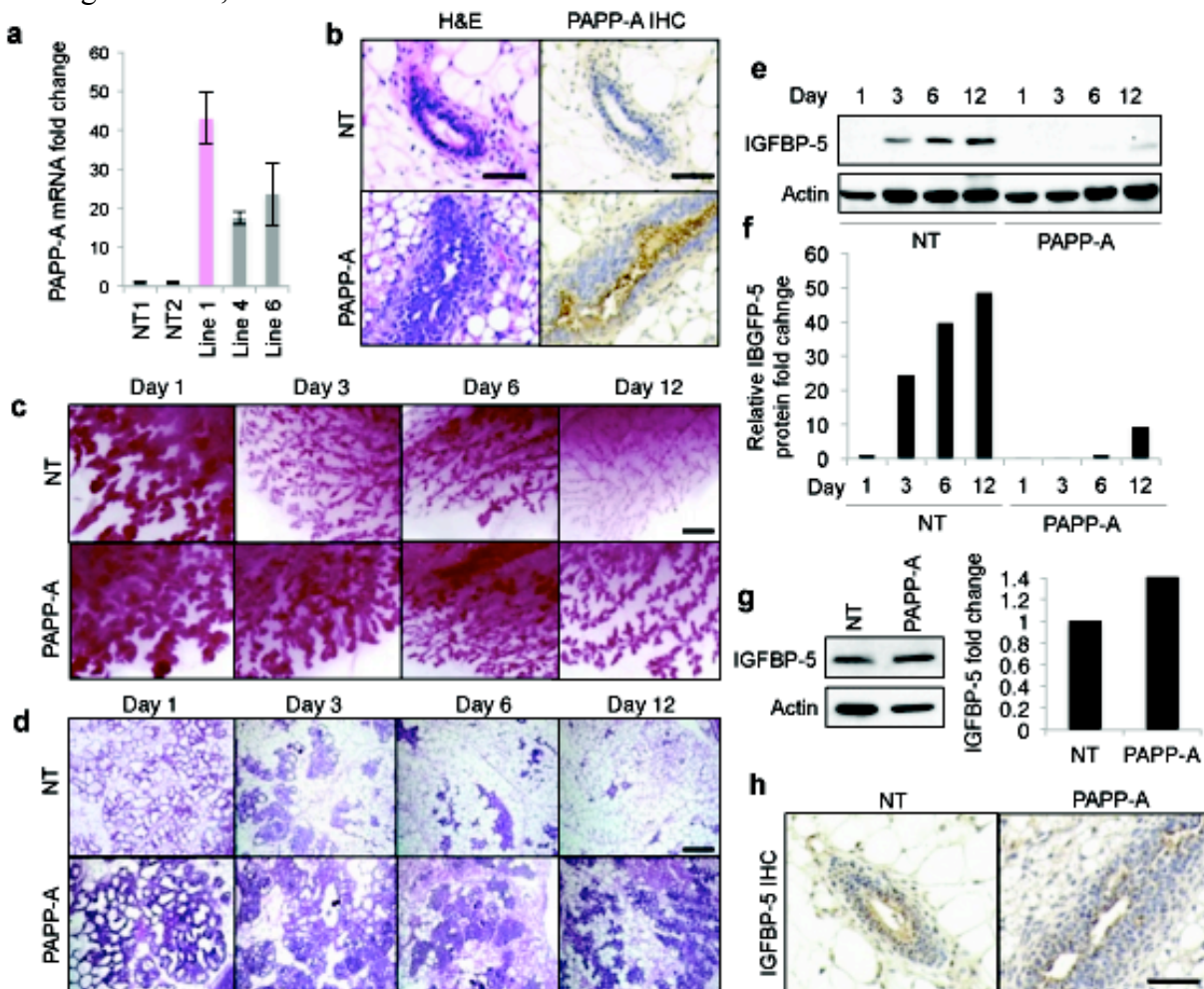


Figure 3 Increased collagen deposition is observed during mammary gland involution in PAPP-A transgenic mice and enhances the proteolytic activity of PAPP-A. **(a)** Masson's trichrome stain (MTS) of involuting mammary glands of NT and PAPP-A transgenic mice. Blue: collagen. Scale bar: 100um. **(b)** Second harmonic generation (SHG) imaging of collagen on histological sections shown in (a). Scale bar: 100um. **(c)** Quantification of collagen by MTS from (a). **(d)** Graph of collagen intensity versus distance from NT and PAPP-A involuted mammary ducts as imaged by SHG in (b). **(e)** 50ng Recombinant IGFBP-5 (rIGFBP-5) fractionated into supernatant (S) and pellet (P) in the presence of media, collagen (+Col-1), collagen neutralizing solution (+ N.S.), or detergent (+NP40). **(f)** Protein levels (in S versus P fractions) of 50ng rIGFBP-5 incubated for 3 hours in control media from MCF7 parental cells or PAPP-A media taken from stable MCF7^{PAPP-A} clones, in the absence or presence of collagen. **(g)** Immunoblot and **(h)** quantification of 50ng rIGFBP-5 incubated for 3 hours in culture media from MCF7 parental cells, MCF7 clones stably expressing wild-type PAPP-A (MCF7^{wtPAPP-A}) or proteolytically inactive PAPP-A (MCF7^{mutPAPP-A}) in the absence of collagen. **(i)** Immunoblot and **(j)** quantification in a corresponding assay as in (h) in the presence of collagen. **(k)** Immunoblot and **(l)** quantification of endogenous IGFBP-5 levels of parental MCF7, MCF7^{wtPAPP-A} stable clones or MCF7^{mutPAPP-A} stable clones, in the absence of collagen. **(m)** Immunoblot and **(n)** quantification in a corresponding assay as in (l) in the presence of collagen.

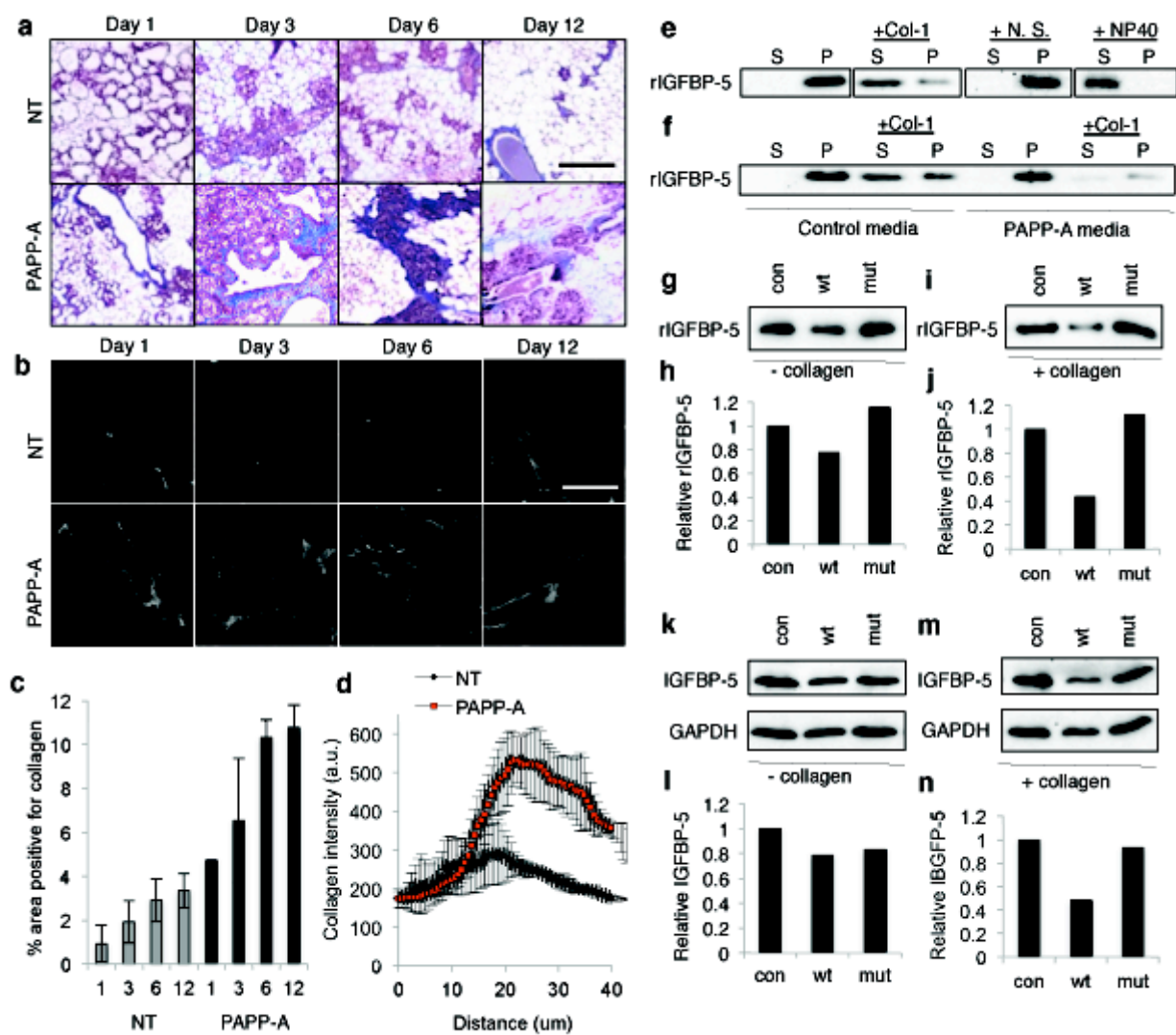


Figure 4 Pregnancy-associated PAPP-A mammary tumors are characterized by low IGFBP-5 levels and a TACS3 signature. (a) Whole mount sections of PAPP-A mammary glands after 1 or 3 pregnancies and PAPP-A age-matched virgin. White arrows indicate lesions. Scale bar: 1mm. (b) Frequency of mammary lesions in NT virgin/pregnancy and PAPP-A virgin/pregnancy glands, n = 4 in each group. (c) Image of a late-stage post-partum PAPP-A mammary tumor before excision. (d) Histological sections of tumor from (c), left: H&E, center: IGFBP-5 immunohistochemistry, right: IGFBP-5 IHC of NT involuted gland. Scale bar: 100um. (e) Relative PAPP-A mRNA in mammary glands of NT virgin, PAPP-A virgin, and a PAPP-A virgin 1 week after injection of estrogen pellet. (f) Relative PAPP-A mRNA in mammary glands of virgin, or involuting glands at day 1 or day 12 in NT versus PAPP-A transgenic females. (g) Imaging of collagen by SHG of virgin PAPP-A ducts or PAPP-A mammary tumor, and mammary tumors from MMTV-HER2 or -WNT transgenic models. Scale bar: 50um. (h) TACS3 analysis for collagen alignment of SHG images from (g).

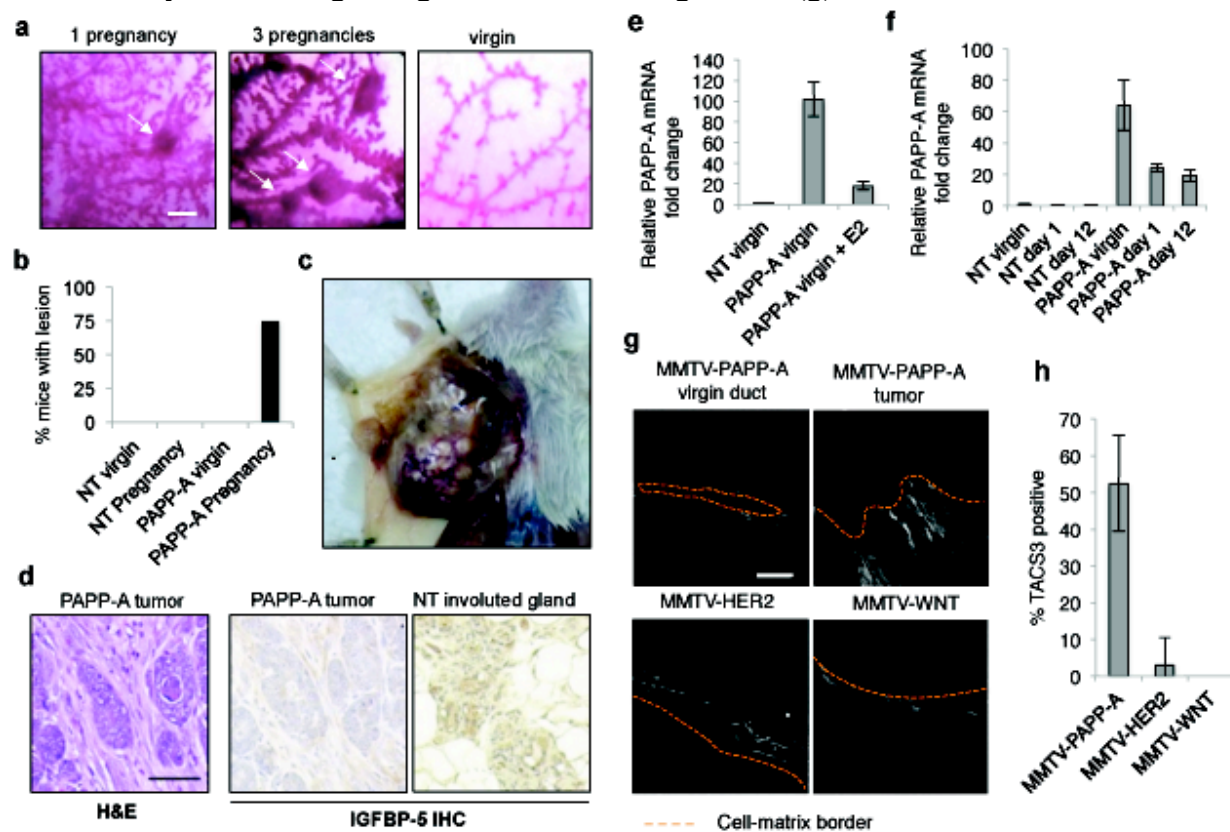


Figure 5 A strong TACS3 signature and decreased IGFBP-5 levels characterize postpartum breast cancers. **(a)** SHG image of collagen of human breast cancers from parous and nulliparous patients. The dotted line indicates the tumor-stroma border. Scale bar: 50um. **(b)** Distribution of collagen curvelet angles, as calculated by CurveAlign software, of breast cancer sections from nulliparous (n=9) and parous (n=14) patients. **(c)** TACS3 signature score of breast cancer from nulliparous (n = 9) and parous (n = 14) patients, $p < 0.0001$. **(d)** Representative scoring of immunohistochemistry of IGFBP-5 on invasive breast cancer sections, with score 0 as negative staining, score 1 as weak staining, and score 2 as strong staining. Top scale bar: 100um. Magnified image scale bar: 20um. Table displays distribution of score in nulliparous (n = 10) and parous (n = 12) sections. **(e)** Schematic diagram of a mechanism by which upregulated PAPP-A cleaves IGFBP-5 (left) and increases binding of IGF to IGF receptors, resulting in increased IGF signaling. In addition to maintaining proliferation, elevated IGF signaling promotes the synthesis of collagen (right), which in turn facilitates IGFBP-5 proteolysis by PAPP-A, establishing a positive feedback loop. Since increased deposition of collagen in breast cancers from parous women is characterized by a TACS3 signature, which stimulates invasion, we hypothesize that low IGFBP5 and strong TACS3 contribute to the more aggressive nature of PABC.

